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# Functional regulatory T cells are Collected in Stem Cell Autografts by Mobilization with High-Dose Cyclophosphamide and Granulocyte Colony-Stimulating Factor

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**Running title:** Regulatory T lymphocytes in stem cell autografts

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**Abstract**

High-dose cyclophosphamide (Cy) and G-CSF are widely used to mobilize hematopoietic stem cells (HSC) for treating patients with high dose chemotherapy and autologous stem cell transplantation (ASCT). As lymphocyte count in the graft collected after Cy-G-CSF treatment is an independent survival factor after ASCT for patients with multiple myeloma (MM), our purpose was to study how Cy-G-CSF treatment affects the phenotype and function of T cells in patients with MM. Cy induced a threefold decrease of T-cell counts with a slow and partial T-cell recovery of one-third at the time of HSC collection. Cy-G-CSF treatment did not affect the relative ratios of central memory, effector memory, and late effector CD4$^+$ or CD8$^+$ T cells, but a decrease in the percentage of naive CD4$^+$ cells was observed. The percentages of CD25$^+$ cells increased two- to threefold in CD4$^+$ and CD8$^+$ T cells, the former including both activated CD25$^{low}$ and CD25$^{high}$ cells. CD4$^+$CD25$^{high}$ cells were regulatory T cells (Treg) that expressed high levels of *FOXP3*, *CTLA-4* and *GITR* and displayed *in-vitro* suppressive properties. The recovery of Treg absolute counts post Cy-G-CSF treatment was higher than the recovery of other lymphocyte subpopulations. In conclusion, Cy-G-CSF treatment induces a severe T-cell count decrease without deleting Treg, which are potent inhibitors of anti-tumor response. The present data encourage novel therapeutic strategies to improve T-cell recovery following ASCT while limiting Treg expansion.

**Introduction**

High-dose chemotherapy (HDC) associated with autologous stem cell transplantation (ASCT) improves the rate of complete remission and overall survival in patients with multiple myeloma (MM) and non-Hodgkin lymphoma at relapse (1, 2). Many studies have focused on the efficient mobilization of hematopoietic stem cells (HSC) by administration of hematopoietic growth factors with or without high-dose cyclophosphamide (Cy) (3-8). Cy induces profound aplasia with the production of endogenous mediators and growth factors that favor hematopoietic recovery. These endogenous mediators and the administrated recombinant granulocyte colony-stimulating factor (G-CSF) stimulate HSC proliferation and their mobilization into the peripheral blood. This mobilization is attributable to the activation of bone marrow neutrophils by hematopoietic growth factors, in particular G-CSF, resulting in the production of enzymes which cleave adhesion molecules and chemokines and in the release of HSC from the bone marrow environment (9, 10). Because T lymphocytes are major actors in the graft-versus-host reaction in allogenic HSC transplantation, the characteristics of T lymphocytes collected by leukapheresis after HSC mobilization with G-CSF have been analyzed. Some studies indicate that G-CSF doubles the circulating T lymphocyte rate without major changes in the phenotype of T cells (11, 12). Several other studies (13-15) demonstrate that G-CSF might affect the cytotoxic T-cell activity by inducing human lymphocytes to preferentially release type 2 cytokines (IL-4, IL-5, and IL-10) rather than type 1 (IFN$\gamma$, TNF$\alpha$ and IL-2) upon activation. However, the phenotype and function of T lymphocytes mobilized with Cy and G-CSF have not been studied yet. This is important because Cy is a strong immunosuppressor. In mice, the Cy-induced immunosuppression is followed by a rapid T-cell repopulation, likely associated with the production of endogenous T-cell growth factors (16). In addition, a selective toxicity of Cy on regulatory T cells (Treg) has been demonstrated (17-19). Treg are CD4^{+}CD25^{+} T cells

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that control key aspects of tolerance to self-antigens by suppressing activation of naïve T cells and they are crucial in the prevention of autoimmune diseases (20, 21). Treg express a specific transcription factor, *FOXP3*, which controls both their development and function (22). They also constitutively express some molecules associated with T-cell activation including GITR and CTLA-4 (23, 24). Because a part of tumor-associated antigens is derived from self-antigens, Treg may be partially responsible for the lack of anti-tumor immune responses. In animal models, Treg expansion in the lymphoid organs of tumor-bearing animals is correlated with tumor volume, and the removal of Treg enhances anti-tumor immune responses. In humans, the proportion of Treg is increased in cancer patients. Thus, the depletion of Treg could be useful to promote an anti-tumor T-cell response (20, 21).

A recent study has emphasized that the number of infused autologous lymphocytes collected by Cy and hematopoietic growth factor mobilization was an independent factor for overall survival and time to progression post HDC and ASCT, in patients with MM (25). Given the lack of data regarding the phenotype and function of T lymphocytes in the HSC graft, the aim of our study was to characterize the behaviour of T-cell subpopulations after Cy-G-CSF treatment, and in particular that of Treg. Human $CD4^+$ or $CD8^+$ T cells can be classified into naïve T cells, central memory T cells, effector memory T cells, and late effector T cells (26, 27). During an immune reaction against a pathogen, naïve T cells are primed by antigen-loaded dendritic cells in lymphoid organs, proliferate widely and differentiate into effector cells, which produce cytokines and are able to kill pathogens. After the acute activation phase, a fraction of the activated T lymphocytes persists as central memory T cells, able to induce a quick and strong immune response upon reencountering antigen. Naïve T cells express CD45RA and two lymph-node homing receptors, CD62L, which enables T cells to adhere to endothelial cells, and CCR7, which is a receptor for the CCL19 and CCL21 lymph-node chemokines. Central memory T cells are $CD45RA^-$ and express the chemokine

receptors CCR7 and CD62L. Effector memory T cells are CD45RA⁻ and lack CCR7 and CD62L but express receptors for inflammatory chemokines (CCR1, CCR3, and CCR5). Late effector T cells are CD45RA$^+$ CCR7$^-$ with a low expression of CD62L. In healthy individuals, there are very few circulating late effector CD45RA$^+$CCR7$^-$ CD4$^+$ T cells compared to the high proportion of late effector CD8$^+$ T cells (28).

We investigated here the phenotype and function of T lymphocytes present in the peripheral blood after high-dose Cy-induced aplasia and G-CSF mobilization in 14 patients with multiple myeloma.

**Materials and Methods**

*Patients and collection of peripheral blood samples*

Fourteen patients with MM (median age: 59 years) who underwent ASCT were included in this study, according to the French ethical laws. The series comprised nine male patients and five female patients. According to the Durie-Salmon classification, ten patients had stage IIIA disease, two stage IIIB, while one presented with a plasma cell leukemia and one patient had a recurring plasmacytoma. One patient had IgAκ MM, one IgAλ MM, three IgGλ MM, eight IgGκ MM and one Ig non-secreting plasmacytoma. The mobilization procedure consisted of a single 4-g/m$^2$ Cy infusion followed by daily subcutaneous injections of 10 μg/kg/day G-CSF, until completion of HSC collection ($\geq 6 \times 10^6$ CD34$^+$ cells/kg) by leukapheresis. Blood samples were collected after written informed consent, on the day before Cy administration (day 0), 6 days after Cy administration (nadir) and on the day of the first leukapheresis procedure (median day 10; range: 9-12). Peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation using Ficoll-Hypaque (Cambrex BioScience, Walkersville, MD). After written informed consent, $500 \times 10^6$ fresh cells from leukapheresis products were used for Treg sorting and functional assays.

*Flow cytometry analysis*

The phenotype of T cells was evaluated with the following monoclonal antibodies (Abs): phycoerythrin (PE)-conjugated anti-CD3, anti-CD4, anti-CD8β, anti-CD25 and anti-panγδTCR; fluorescein isothiocyanate (FITC)-conjugated anti-CD25, anti-CD4 and anti-CD8 (Beckman Coulter, Villepinte, France) and anti-CCR7 (R&D System, Abington, UK); PE-Cy5-conjugated anti-CD45RA and anti-CTLA-4 (BD Biosciences Pharmingen, San Jose, CA); APC-conjugated anti-GITR (BD Biosciences Pharmingen). The phenotype of NK cells was evaluated with a PE-conjugated anti-CD56 Ab (Beckman Coulter). Corresponding isotype-matched murine Abs, recognizing no human antigen, were used as negative controls.

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Briefly, appropriate amounts of Abs were added to $0.5 \times 10^6$ cells followed by a 30-min incubation at 4°C. Red cells were then lysed, cells were washed and $30 \times 10^4$ total events or $10 \times 10^4$ events in the lymphocyte gate were acquired on a FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA) or on a FACSAria™ cell sorter (Becton Dickinson). Analyses were performed with the CellQuest software. Lymphocyte subsets were assessed by three-color immunofluorescence analysis. For determination of intracellular expression of CTLA-4, cells already stained for CD4 and CD25 were fixed for 20 min at 4°C with the Cytofix/Cytoperm kit (BD Biosciences Pharmingen). Intracytoplasmic staining was then performed at 4°C in saponin-containing perm/wash buffer and in the presence of PE-Cy5-conjugated anti-CTLA-4 Ab.

*T cell purification*

$CD4^+CD25^{high}$, $CD4^+CD25^{low}$ and $CD4^+CD25^-$ T cells were purified from fresh PBMCs harvested before Cy-G-CSF treatment or from leukapheresis product. $CD4^+$ T cells were first enriched by depletion of monocytes and B cells with anti-CD14 and anti-CD19 Abs-coated magnetic beads (Dynal, Oslo, Norway). Then anti-CD25-PE and anti-CD4-FITC Abs were added and $CD4^+CD25^-$, $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ cells were sorted using a FACSAria™ cell sorter (Becton Dickinson). The purity of the T-cell subpopulations was greater than 90%.

*Quantification of FOXP3, CTLA-4 and GITR by real-time RT-PCR*

Total RNA was prepared from sorted $CD4^+$ subpopulations using the RNeasy Kit (Qiagen, Hilden, Germany). cDNA was prepared from 1 µg of RNA using the Superscript II reverse transcriptase (RT; Invitrogen, Cergy Pontoise, France) and oligo $d(T)_{12-18}$ (Amersham Pharmacia Biotech, Orsay, France) as primer. The relative levels of *FOXP3*, *CTLA-4* or *GITR* mRNA in each subset were determined by real-time PCR on an ABI Prism 7000 Sequence Detector (Applied Biosystems, Courtaboeuf, France) using the Assays-On-Demand products

(Hs00203958_m1 for *FOXP3* detection; Hs00175480_m1 for *CTLA-4* detection and Hs00188346_m1 for *GITR* detection ) and the *Taq*Man Universal Master Mix. Quantitative PCR analysis was completed using ABI Prism 7000 SDS Software. Threshold cycles (Ct) values were measured for beta-2-microglobulin (*β2m*) and the genes of interest during the log phase of the cycle. The expression levels of genes of interest were normalized to that of *β2m* for each sample (δCt = Ct gene of interest – Ct *β2m*) and compared with the values obtained for a CD4$^+$CD25$^{high}$ positive control isolated from a healthy donor, using the following formula: $100/2^{δδCt}$ where δδCt = δCt unknown – δCt positive control.

*Treg suppression assay*

Mature dendritic cells (DCs) were generated as detailed previously (29). Briefly, $8 \times 10^6$ G-CSF-mobilized leukapheresis cells were plated in 2 mL of X-VIVO15 medium (BioWittaker, Walkersville, MD) per well in six-well flat-bottomed plates (Nunc, Roskilde, Denmark). Nonadherent cells were discarded by gentle rinsing after a 2-h incubation at 37°C in 5% $CO_2$. Adherent cells were cultured in X-VIVO15 medium with 2% human albumin, 100 ng/mL of GM-CSF (LEUKINE$^®$, Berlex, Montville, NJ) and 25 ng/mL of IL-4 (Cellgenix, Freiburg, Germany) for 5 days. DCs were then induced to mature for 24 h with 20 ng/mL of TNFα (Cellgenix) and 100 ng/ml of PGE2 (PROSTIN E2$^®$, Pharmacia, Kalamazoo, MI) and finally frozen in X-VIVO15-50% albumin-10% DMSO medium.

In each well, $10^4$ CD4$^+$CD25$^-$ T cells, CD4$^+$CD25$^{low}$ T cells or CD4$^+$CD25$^{high}$ T cells were seeded into 96-well U-bottom culture plates in a 200-µL final volume of RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% human serum with or without $10^3$ allogeneic mature DCs. Either $10^4$ CD4$^+$CD25$^{high}$ T cells or $10^4$ CD4$^+$CD25$^{low}$ T cells were added to wells containing DC-stimulated CD4$^+$CD25$^-$ T cells. At the end of a 7-day culture, cells were pulsed with tritiated thymidine (Amersham Pharmacia Biotech) for 16 h, and tritiated thymidine incorporation was determined as reported previously (30).

**Results**

*Mobilization of T and NK lymphocytes*

T-cell and NK-cell subpopulations profiles were studied for fourteen patients. Mean white blood cell (WBC) count dropped on day 6 following Cy administration and then increased due to G-CSF injection (Fig. 1*A*). The mean WBC count was $10 \times 10^9$/L on the day of the first leukapheresis procedure (median day 10, range: 9–12). G-CSF injections were stopped after HSC collection. Cy induced a sevenfold reduction of the CD3$^+$ cell count on day 6 followed by a slow and partial recovery in all 14 patients (Fig. 1*B*). The mean CD3$^+$ cell count was $1.22 \times 10^9$/L before Cy-G-CSF treatment and dropped to $0.18 \times 10^9$/L six days after Cy injection, corresponding to the WBC count nadir. It then increased slightly to a mean count of $0.36 \times 10^9$/L on the first leukapheresis procedure day. The relative ratios of CD4$^+$ and CD8$^+$ cells within CD3$^+$ cells were not significantly modified throughout the Cy-G-CSF-induced mobilization period (results not shown). NK cells counts paralleled the decrease of CD3$^+$ cells with a one-fifth partial recovery on the first leukapheresis procedure day (Fig.1*C*). Thus Cy administration induced a profound decrease of CD3 and NK cell counts.

*Mobilization of naïve, central memory, effector memory and late effector T cells*

We studied the subpopulations of naïve CD4$^+$ T cells (CD45RA$^+$, CCR7$^+$), central memory CD4$^+$ T cells (CD45RA$^-$, CCR7$^+$), effector memory CD4$^+$ T cells (CD45RA$^-$CCR7$^-$), and late effector CD4$^+$ T cells (CD45RA$^+$ CCR7$^-$) throughout the Cy-G-CSF mobilization period in ten patients with MM. Given the large amount of data, only the mean percentages of CD4$^+$ or CD8$^+$ subsets are shown in Fig. 2 *A* and *B*. Before Cy-G-CSF treatment, the mean percentages of the various subpopulations of CD4$^+$ cells were: naïve CD4$^+$ cells: 28% (range 14–45%), central memory CD4$^+$ cells: 47% (range 35.7–65%), effector memory CD4$^+$ cells: 21% (range 12.7–31%), and late effector CD4$^+$ cells: 5% (range 0.1–16.3%) (Fig. 2*A*). This

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profile of CD4$^+$ cell subpopulation distribution did not differ from those previously reported for a healthy donor population (respectively, 28%, 58%, 12%, and 2%) (31). We found that Cy-G-CSF treatment induced a slight but significant ($P < .05$) decrease in naïve CD4$^+$ cells relative rate (from 28% to 19%), while the percentages of the three subpopulations of memory CD4$^+$ cell (central memory, effector memory, and late effector) were not significantly affected (Fig. 2*A*). Before Cy-G-CSF treatment, CD8$^+$ T cells comprised 25% (range 12.3–49%) of naïve cells, 14% (range 6.7–27.5%) of central memory cells, 23% (range 5.7–40%) of effector memory cells and 38% (range 18.6–64%) of late effector cells. The proportions of CD8$^+$ subsets did not change significantly throughout Cy-G-CSF-induced HSC mobilization (Fig. 2*B*) and did not differ from those previously reported in healthy donors (respectively, 30%, 8%, 30% and 32%) (32).

*Regulatory and activated T cells*

The proportions of CD4$^+$ and CD8$^+$ T cells that expressed CD25 increased throughout Cy-G-CSF mobilization. For one representative patient, a dot blot of CD25 labelling of CD4$^+$ and CD8$^+$ cells in the peripheral blood before mobilization and on the day of leukapheresis is shown in Fig. 3*A*. The mean values of the 14 patients are shown in Fig. 3*B*. A mean percentage of 46% of CD4$^+$ T cells and of 13% of CD8$^+$ T cells expressed CD25 on the day of the first leukapheresis procedure (median day 10, after Cy administration, range 9-12 days) (Fig. 3*B*). These mean percentages were significantly increased 2.0- and 2.9-fold respectively, compared to those observed before Cy-G-CSF treatment ($P \leq .01$). In humans, CD4$^+$CD25$^+$ cells contain both regulatory T cells that express high levels of CD25 (CD4$^+$CD25$^{high}$) and activated non-regulatory CD4$^+$CD25$^{low}$ cells (33). These two populations could be found before but also after Cy-G-CSF treatment, suggesting that Treg are still present after Cy administration (Fig. 4*A*). The mean percentages of both CD4$^+$CD25$^{high}$ and CD4$^+$CD25$^{low}$ subsets were increased 2-fold ($P \leq .01$) at the time of HSC

collection compared to those observed before Cy-G-CSF treatment (Figs. 4*B* and *D*). The mean CD4$^+$CD25$^{high}$ and CD4$^+$CD25$^{low}$ cell counts were decreased 5.6-fold and 3.6-fold respectively ($P \leq .01$) on day 6 after Cy administration. At the time of HSC collection, the mean CD4$^+$CD25$^{high}$ and CD4$^+$CD25$^{low}$ cell counts did not significantly differ from those prior Cy-G-CSF treatment, contrary to CD3$^+$ cell or NK cell counts which remained at least 3-fold decreased (Fig. 1*B-C*). As these data are intriguing given the reported toxicity of Cy to Treg (17, 18, 34), the phenotype and function of CD4$^+$CD25$^{high}$ cells in the leukapheresis product were studied in detail. CD4$^+$CD25$^{high}$, CD4$^+$CD25$^{low}$ and CD4$^+$CD25$^-$ cells contained in the leukapheresis products from six patients with MM were purified using a FACSAria™ cell sorter (Fig. 5*A*). *FOXP3, CTLA-4* and *GITR* gene expressions were assayed using real-time RT-PCR. *FOXP3* gene expression in CD4$^+$CD25$^{high}$ cells was 60- to 200-fold higher than in CD4$^+$CD25$^-$ ($P < .001$) cells and 6- to 20-fold higher than in CD4$^+$CD25$^{low}$ cells ($P < .01$) (Fig. 5*B*). CD4$^+$CD25$^{high}$, CD4$^+$CD25$^{low}$ and CD4$^+$CD25$^-$ cells contained in the peripheral blood before mobilization from 4 patients with MM were also purified. A similar pattern of *FOXP3* expression was found in the three purified subpopulations (data not shown). CD4$^+$CD25$^{high}$ cells harvested before (data not shown) and on the day of leukapheresis (Fig. 5*C-D*) also overexpressed the *CTLA-4* ($P = .03$) and *GITR* ($P = .05$) genes, other markers associated with Treg function. The expressions of *CTLA-4* and *GITR* were confirmed at the protein level. As shown in Fig. 5*E* for one representative patient out of 5 patients with MM, CD4$^+$CD25$^{high}$ cells were strongly labeled with anti-CTLA-4 Ab, unlike CD4$^+$CD25$^-$ cells. CD4$^+$CD25$^{low}$ cells displayed an intermediate CTLA-4 expression. CD4$^+$CD25$^{high}$ cells collected on the day of leukapheresis also expressed higher level of GITR than CD4$^+$CD25$^{low}$ or CD4$^+$CD25$^-$ cells. A similar high GITR and CTLA-4 protein expression by CD4$^+$CD25$^{high}$ cells harvested before the mobilization was found for 5 patients with MM (data not shown).

To prove definitively that CD4$^+$CD25$^{high}$ cells were Treg, we studied their ability to inhibit the activation of autologous CD4$^+$CD25$^-$ cells by allogeneic DCs. CD4$^+$CD25$^{high}$ cells harvested either before or after Cy administration abrogated activation of CD4$^+$CD25$^-$ cells induced by allogeneic DCs, at 1:1 ratio of CD4$^+$CD25$^-$ cells to Treg cells (Fig. 6). CD4$^+$CD25$^{low}$ cells were efficiently stimulated by DCs, contrary to CD4$^+$CD25$^{high}$ cells and were unable to inhibit activation of CD4$^+$CD25$^-$ cells (Fig. 6). Altogether these data clearly demonstrate that circulating CD4$^+$CD25$^{high}$ cells present during Cy-G-CSF mobilization are functional Treg cells, as are circulating CD4$^+$CD25$^{high}$ cells harvested before Cy-G-CSF treatment, whereas the CD4$^+$CD25$^{low}$ cells are activated non-regulatory T cells.

*Immune cell composition of leukapheresis products*

In Table I is depicted the cell composition of leukapheresis products of the 14 patients with MM. These products contained median values of $6.2 \times 10^6$ CD34$^+$ cells/kg (range: 2.68‑9.1), $21.1 \times 10^6$ CD3$^+$ cells/kg (range: 13‑116), including $15.8 \times 10^6$ CD4$^+$ cells/kg and $6.3 \times 10^6$ CD8$^+$ cells/kg, $3.8 \times 10^6$ NK cells/kg (range: 1.5‑46.9) and $0.3 \times 10^6$ γδ T cells/kg (range: 0.1‑5.3). They contained nearly $1 \times 10^6$ CD4$^+$CD25$^{high}$ cells/kg (range: 0.4‑5.1) with the phenotype and suppressive properties of Treg. The ratio of CD34+ cells to Treg ranged from 13.4 to 0.5 for a median value of 7.

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**Discussion**

Mobilization regimens have been optimized to collect a maximum of CD34$^+$ HSC in order to reduce aplasia and the number of leukaphereses. High-dose Cy associated with G-CSF is widely used because it results in a two- to three-fold increase in the number of CD34$^+$ HSC collected per leukapheresis procedure compared to G-CSF alone (7, 8). However, very little attention has been given to the impact of Cy-G-CSF combination on the number, phenotype and function of the T cells that are collected in the leukapheresis product and infused to the patient.

In a cohort of 14 consecutive patients with MM, we show that Cy-G-CSF treatment induced a severe T-cell and NK-cell counts decrease. Circulating CD3$^+$ cell counts were reduced sevenfold on day 6 after Cy administration and slowly and partially recovered, contrary to a rapid recovery of neutrophil and platelet counts. On the contrary, mobilization with G-CSF alone was shown to double circulating CD3$^+$ cell counts (11, 12). Thus, although Cy increases the mobilization and collection of CD34$^+$ cells, it dramatically reduces the number of circulating T cells. The effect of the mobilization regimen on T-cell count might be of importance since Porrata *et al*. have recently shown that absolute lymphocyte count in the autograft collected after Cy and hematopoietic growth factors treatment may affect survival in MM patients after ASCT (25). Patients receiving a high number of lymphocytes had higher response rates and lower relapse rates, resulting in improved survival. In particular, the autograft absolute lymphocyte count was an independent prognostic factor for overall survival and progression-free survival in MM patients (25). In this study, the lymphocyte population was not characterized and may include T cells, B cells, NK cells or eventually non-lymphocyte cells.

T cells comprise several subsets with distinct phenotype and function. Based on CD45RA and CCR7 chemokine receptor expression, we investigated four CD4$^+$ or CD8$^+$ T-cell

subpopulations: naïve T cells (CD45RA$^+$, CCR7$^+$), central memory T cells (CD45RA$^-$, CCR7$^+$), effector memory T cells (CD45RA$^-$, CCR7$^-$) and late effector T cells (CD45RA$^+$, CCR7$^-$). Before Cy-G-CSF treatment, the proportions of the four CD4$^+$ subsets and the four CD8$^+$ subsets in patients with MM were similar to those previously described in healthy individuals. They remained stable throughout the partial T-cell recovery after Cy administration, except for a slight decrease in the naïve CD4$^+$ subset. Thus, the current data indicate that the Cy-G-CSF mobilization regimen, while inducing a severe reduction in T-cell count, did not significantly affect the proportions of memory and naïve T cells. This is important considering that the memory T-cell repertoire in the autograft is critical to T-cell immunity reconstitution after high-dose chemotherapy (35).

We then investigated T-cell activation. The percentages of CD25$^+$ cells in CD4$^+$ or CD8$^+$ cells increased two- or three-fold throughout post-Cy lymphocyte recovery. In healthy individuals or patients with MM, CD4$^+$CD25$^+$ cells include regulatory T cells that highly express CD25 (CD4$^+$CD25$^{high}$ cells) and activated non-regulatory CD4$^+$ T cells (CD4$^+$CD25$^{low}$ cells). The current study indicates that the percentages of both circulating subpopulations doubled during Cy-G-CSF mobilization. Concerning CD4$^+$CD25$^{low}$ cells, such a high proportion of activated cells (40% of CD4$^+$ cells) was not reported in studies that explored the phenotype of G-CSF mobilized T cells (12, 14, 36). This may be explained by an *in vivo* production of a cytokine burst able to activate T cells, that occurs after Cy treatment. In particular, Proietti *et al*. demonstrated in a murine model that one of these mediators was interferon-alpha (16, 37).

We demonstrated that CD4$^+$CD25$^{high}$ cells collected in the leukapheresis product have the phenotype of Treg. They highly express the FOXP3, CTLA-4 and GITR genes and the CTLA-4 and GITR proteins compared to CD4$^+$CD25$^{low}$ or CD4$^+$CD25$^-$ cells. FOXP3 is one of the best Treg specific marker available to date (22). CTLA-4 is usually upregulated on T cells after activation. It has been shown to be constitutively expressed by Treg and is involved

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in their suppressive function (24). GITR (glucocorticoid-induced TNF receptor), a member of the tumor necrosis factor receptor superfamily, is a costimulatory molecule overexpressed on Treg but also found on activated T cells. Activation of GITR may abrogate Treg suppressive function (23, 38).

We also demonstrated that CD4$^+$CD25$^{high}$ cells collected in the leukapheresis product strongly suppress the proliferation of autologous CD4$^+$CD25$^-$ cells induced by allogeneic DCs, unlike CD4$^+$CD25$^{low}$ cells. Thus, CD4$^+$CD25$^+$ cells in the leukapheresis product contain both activated lymphocytes and functional Treg, and the frequency of Treg in CD4$^+$ cells is doubled at the time of HSC collection compared to pre-Cy-GSF treatment.

This observation is noteworthy because low doses of Cy have a specific toxicity to Treg in rodents, leading to Treg apoptosis and loss of function (17-19, 34, 39). In particular, Ghiringhelli *et al*. demonstrated that a single injection of a low dose of Cy in tumor-bearing rats abrogated the rise of Treg that generally occurred during tumor progression, resulting in the restoration of an anti-tumor immune response (18). More recently, Lutsiak *et al*. demonstrated that in addition to decreasing the Treg cell number, a low dose of Cy inhibits the suppressive capability of Treg in mice (34). The fact that Treg are still present and fully functional after mobilization with high-dose Cy and G-CSF could be assigned to G-CSF because it increases the number of circulating Treg in healthy individuals (40). Furthermore, Hadaya *et al*. have demonstrated that G-CSF treatment prevents Cy acceleration of autoimmune diabetes in NOD mice by stimulating the expansion of regulatory CD4$^+$CD25$^+$CD62L$^{high}$ T cells (41). It has also been reported in a NOD mouse model that G-CSF injection promotes the splenic accumulation of plasmacytoid tolerogenic DCs, which have the property to expand Treg (42). Our data indicate that the median Treg count in leukaphereses is approximately one-seventh that of CD34$^+$ cells. As more than $3 \times 10^6$/kg CD34$^+$ cells are generally grafted to patients, more than $4 \times 10^5$/kg Treg are also grafted.

These Treg could contribute to hindering the reconstitution of T cells after HDC. In particular, given the well-documented suppressive activity of Treg on anti-tumor T cells (43-45), these grafted Treg could affect the emergence of anti-myeloma T cells after HDC. In this small series of 14 patients with MM, we found no relationship between the grafted Treg counts and the response rate to HDC and the follow-up of these patients is as yet too short to evaluate a link between Treg count and event-free or overall survival. Our study points out the interest to monitor grafted T cell subsets and Treg counts on a large series of patients to look for their impact on response, event-free and overall survival rates.

The present data could be useful to improve the treatment of patients with MM using high-dose melphalan supported by autologous stem cell transplantation. Indeed high-dose melphalan induces a profound and long-lasting T-cell immunosuppression (46). As we show here that the grafted T cells comprise activated T cells, one can suggest that these patients could be provided with T-cell growth factors to favor T-cell recovery and growth. According to the recent data indicating that grafted lymphocyte counts could predict survival in patients with MM (25), this may dramatically improve treatment response. IL-2 would have a limited interest because (a) systemic IL-2 administration induces severe toxicity, (b) IL-2 is necessary to the homeostatic maintenance of the pool of natural Treg (47) and (c) IL-2 is required to expand Treg cells *in vitro* and to induce their suppressive properties (48). IL-7 or IL-15 could be more promising in a post-transplant setting. Indeed, these two cytokines have reduced systemic toxicities compared to IL-2 injections (49) and they are able to abrogate the suppressive activity of Treg (50). An alternative strategy would be to inject IL-7 and/or IL-15 in patients throughout the Cy-G-CSF-induced mobilization in order to speed up T-cell recovery *in vivo* and to collect high T-cell numbers through apheresis. A third possibility would be to deplete CD25$^{high}$ Treg and then amplify collected T cells with anti-CD3 and anti-CD28-coated microbeads *ex vivo* in order to prepare large numbers of T cells that could be

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grafted after high-dose chemotherapy (51). In particular, Powell *et al*. successfully depleted Treg from leukapheresis products using a clinical-grade large-scale immunomagnetic system (52).

Mobilization regimens to collect HSC in patients with MM are heterogeneous, including Cy and G-CSF, G-CSF alone, cytokine combination (SCF and G-CSF or EPO and G-CSF) (53-55) or the novel AMD3100 CXCR4 inhibitor (56). Only the ability to mobilize the CD34^{+} progenitors and the collection of CD34^{+} cells leukapheresis are generally considered. Our study indicates that the mobilization regimens should also be compared for their ability to affect the number and function of T cells that can be collected and grafted to patients.

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**Table I: Cell composition of infused leukapheresis products**

| Patient | MNC (x10^8/kg) | CD34+ (x10^6/kg) | CD3+ (x10^6/kg) | CD4+ (x10^6/kg) | CD8+ (x10^6/kg) | NK (x10^6/kg) | γδ T cells (x10^6/kg) | Treg (x10^6/kg) | Ratio CD34+/Treg |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.3 | 5.5 | 20.3 | 12.0 | 7.4 | 5.7 | 0.3 | 0.4 | 13.4 |
| 2 | 1.4 | 7.4 | 13.1 | 8.9 | 4.0 | 1.7 | ND | 0.6 | 12.7 |
| 3 | 2.1 | 5.3 | 25.3 | 16.9 | 8.6 | 11.0 | 1.0 | 0.6 | 8.9 |
| 4 | 0.9 | 7.8 | 18.2 | 12.0 | 6.6 | 3.6 | ND | 0.7 | 11.5 |
| 5 | 0.9 | 9.1 | 27.8 | 18.3 | 8.2 | 4.0 | 0.4 | 0.7 | 12.7 |
| 6 | 0.6 | 5.3 | 20.7 | 17.5 | 3.2 | 2.3 | 0.1 | 0.9 | 5.6 |
| 7 | 0.8 | 6.7 | 20.4 | 12.5 | 3.5 | 2.3 | ND | 1 | 7.1 |
| 8 | 1.5 | 5.4 | 39.1 | 19.9 | 9.5 | 3.4 | ND | 1 | 5.5 |
| 9 | 0.5 | 8.5 | 18.3 | 14.7 | 3.0 | 2.9 | 0.3 | 1.1 | 7.4 |
| 10 | 2.1 | 8.3 | 67.5 | 40.6 | 17.6 | 9.3 | 0.3 | 1.2 | 6.8 |
| 11 | 0.8 | 8.4 | 19.7 | 14.6 | 6.0 | 1.5 | ND | 1.3 | 6.6 |
| 12 | 1.2 | 4.3 | 21.6 | 14.6 | 4.9 | 4.2 | ND | 1.4 | 3.0 |
| 13 | 1.4 | 5.7 | 29.1 | 24.4 | 2.8 | 4.7 | 0.3 | 2.4 | 2.3 |
| 14 | 4.1 | 2.6 | 116.1 | 81.5 | 23.2 | 46.9 | 5.3 | 5.1 | 0.5 |
| **mean** | **1.4** | **6.4** | **32.7** | **22** | **7.8** | **7.4** | **1** | **1.3** | **7.4** |
| **median** | **1.3** | **6.2** | **21.1** | **15.8** | **6.3** | **3.8** | **0.3** | **1** | **7** |

Data represent amounts of each cell type that are infused per autograft per patient. ND: not determined

**Figures legends**

**FIGURE 1: Leukocyte and CD3$^+$ cell counts before and throughout Cy-G-CSF mobilization.**

The panel shows white blood cell (WBC) counts (A), CD3$^+$ cell counts (B) or NK cell (CD3$^-$ CD56$^+$) counts (C) measured on the day before Cy administration (day 0), on day 6 after Cy administration (leukocyte nadir) and on the day of the first leukapheresis procedure (median day 10, range: 9-12) for the 14 patients with MM, treated with Cy and G-CSF. Data of individual patients are represented with specific symbol. Black horizontal lines denote mean values at each time point. **\*** Indicates that the mean value is significantly different ($P \leq .05$) from that before Cy administration, using a Student $t$-test for pairs. **\*\*** Indicates that the mean value is significantly different ($P \leq .05$) from that before Cy administration and from the nadir value at day 6, using a Student $t$-test for pairs.

**FIGURE 2: Subpopulations of naïve and memory CD4$^+$ or CD8$^+$ T cells before and throughout Cy-G-CSF mobilization.**

Results are the mean values of naïve (CD45RA$^+$, CCR7$^+$), central memory (CD45RA$^-$, CCR7$^+$), effector memory (CD45RA$^-$, CCR7$^-$) and late effector (CD45RA$^+$, CCR7$^-$) CD4$^+$ (A) or CD8$^+$ (B) T cells determined at the time points described in Fig. 1, for ten patients with MM. **\*** Indicates that the mean value is significantly different ($P \leq .05$) from that before Cy administration, using a Student $t$-test for pairs. As standard deviations could be high due to interpatient variability, they were not shown.

**FIGURE 3: Percentages of CD25$^+$ lymphocytes within CD4$^+$ or CD8$^+$ T cells.**

A. Representative dot plots showing CD25 expression in CD4$^+$ and CD8$^+$ cells before Cy administration and on the day of the first leukapheresis procedure. Peripheral blood cells were stained with anti-CD25-FITC and anti-CD4-PE or anti-CD8β-PE Abs. Control samples were stained with the corresponding IgG2a-FITC, IgG1-FITC or IgG2a-PE isotype-matched

murine Ab. PBMCs were gated to include only small lymphocytes by forward and side scatter. Numbers on dot plots indicate the percentages of CD25$^+$ cells calculated within CD4$^+$ or CD8$^+$ cells.

B. Increased proportion of peripheral CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T cells throughout the mobilization procedure in 14 patients. Results are mean values ± SD of percentages of CD25$^+$ cells within CD4$^+$ or CD8$^+$ T cells for 14 patients with MM determined before (day 0) and after Cy administration, at the time points described in Fig. 1. * Indicates that the mean value is significantly different ($P \le .05$) from that before Cy administration, using a Student $t$-test for pairs.

**FIGURE 4: CD25$^{high}$ and CD25$^{low}$ cells throughout Cy-G-CSF mobilization.**

A. Representative dot plots showing CD25 expression in CD4$^+$ cells before Cy administration and on the day of the first leukapheresis procedure. Peripheral blood cells were stained with anti-CD25-FITC and anti-CD4-PE Abs. The corresponding IgG2a-PE isotype-matched murine Ab was used as a negative control. PBMCs were gated to include only small lymphocytes by forward and side scatter. CD4$^+$ cells were designated CD25$^{high}$ if CD25 expression exceeded that seen in the CD4$^-$ population (which included CD8$^+$ and NK cells). The percentages indicated are those calculated within CD4$^+$ cells.

B, D. Percentages of CD25$^{high}$ (B) and CD25$^{low}$ (D) cells within CD4$^+$ T cells determined before (day 0) and throughout Cy-G-CSF mobilization, at the time points described in Fig. 1, for 14 patients with MM.

C, E. CD25$^{high}$ (C) and CD25$^{low}$ (E) cell counts determined before (day 0) and throughout Cy-G-CSF mobilization, at the time points described in Fig. 1, for 14 patients with MM.

Data of individual patients are represented with specific symbol. Black horizontal lines denote mean values at each time point. * Indicates that the mean value is significantly different ($P \le .01$) from that before Cy administration (day 0), using a Student $t$-test for pairs. *** Indicates

that the mean value is not different from that before Cy administration but is different ($P \leq$ .05) from the nadir value at day 6, using a Student $t$-test for pairs.

**FIGURE 5: *FOXP3, CTLA-4* and *GITR* are highly expressed by CD4$^+$CD25$^{high}$ T cells.**

A. Purification of CD4$^+$CD25$^{high}$, CD4+CD25$^{low}$ and CD4$^+$CD25$^-$ subsets. Mononuclear cells were depleted of CD14$^+$ cells with anti-CD14-coated magnetic beads and then stained with anti-CD25-PE and anti-CD4-FITC Abs. CD4$^+$CD25$^{high}$, CD4+CD25$^{low}$ and CD4$^+$CD25$^-$ cells were sorted using the indicated gates. The percentages on FACS plots indicate the purity of the sorted cells. Results are representative of ten independent experiments.

B-D Relative *FOXP3* (B), *CTLA-4* (C) and *GITR* (D) expressions were measured by real-time RT-PCR in the three CD4$^+$ subsets purified from leukapheresis products of six patients with MM. *FOXP3, CTLA-4* and *GITR* expressions in each T cell subset were normalized with *β2m* expression and compared to *FOXP3*, *CTLA-4* and *GITR* expressions in a healthy donor's CD4$^+$CD25$^{high}$ cells, which was assigned the arbitrary value of 100. Ct values were collected during the log phase of the cycle. *P* values are indicated when significant differences were detected using a Wilcoxon test.

E. Leukapheresis cells were stained with anti-CD25-PE, anti-CD4-FITC and anti-GITR-APC or anti-CTLA-4-PE-Cy5 Abs. A representative case out of 5 independent experiments is shown revealing that CD4$^+$CD25$^{high}$ T cells express higher levels of intracellular CTLA-4 and surface GITR compared with that of CD4$^+$CD25$^{low}$ or CD4$^+$CD25$^-$ T cells. Numbers on FACS plots indicate the percentages of CTLA-4 or GITR-positive T cells among each population.

**FIGURE 6: CD4$^+$CD25$^+$ cells contain both CD4$^+$CD25$^{high}$ regulatory T cells and CD4$^+$CD25$^{low}$ activated non-regulatory T cells.**

CD4$^+$ T-cell subsets from leukapheresis product (A) or PBMCs harvested before Cy-G-CSF treatment (B) were purified as described in Fig. 5. 10$^4$ sorted CD4$^+$CD25$^-$, CD4$^+$CD25$^{low}$ or CD4$^+$CD25$^{high}$ T cells were cultured in RPMI-5% SAB medium alone or stimulated by 10$^3$

thawed allogeneic dendritic cells (DCs). When indicated, either $10^4$ autologous CD4$^+$CD25$^{high}$ T cells or $10^4$ autologous CD4$^+$CD25$^{low}$ T cells were added in wells containing DCs and CD4$^+$CD25$^-$ cells. Cells were cultured for 7 days and results are the mean value ± SD of tritiated incorporation determined in sixplicate culture wells. *P* values are indicated when significant differences were detected using a Wilcoxon test. Data are representative of three independent experiments.
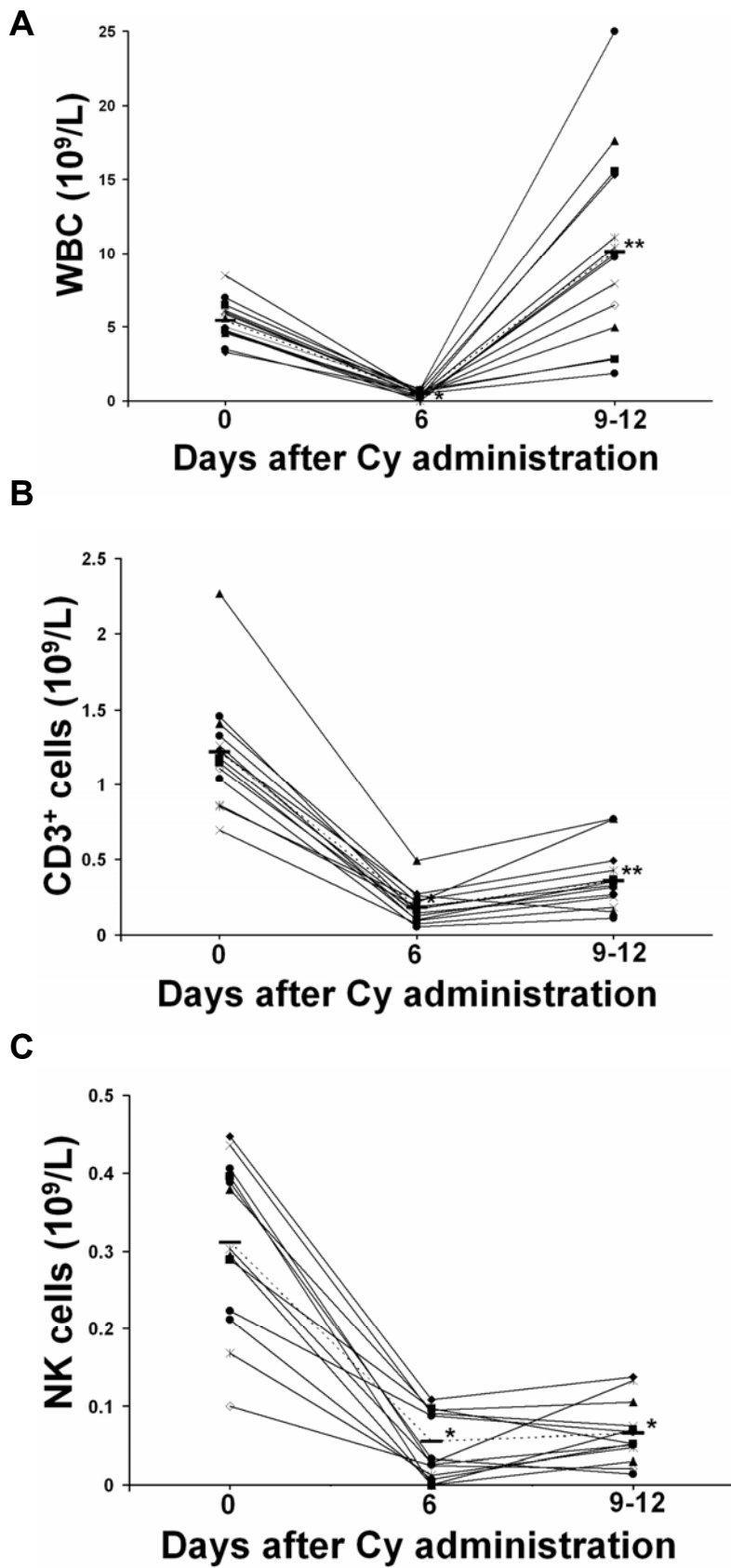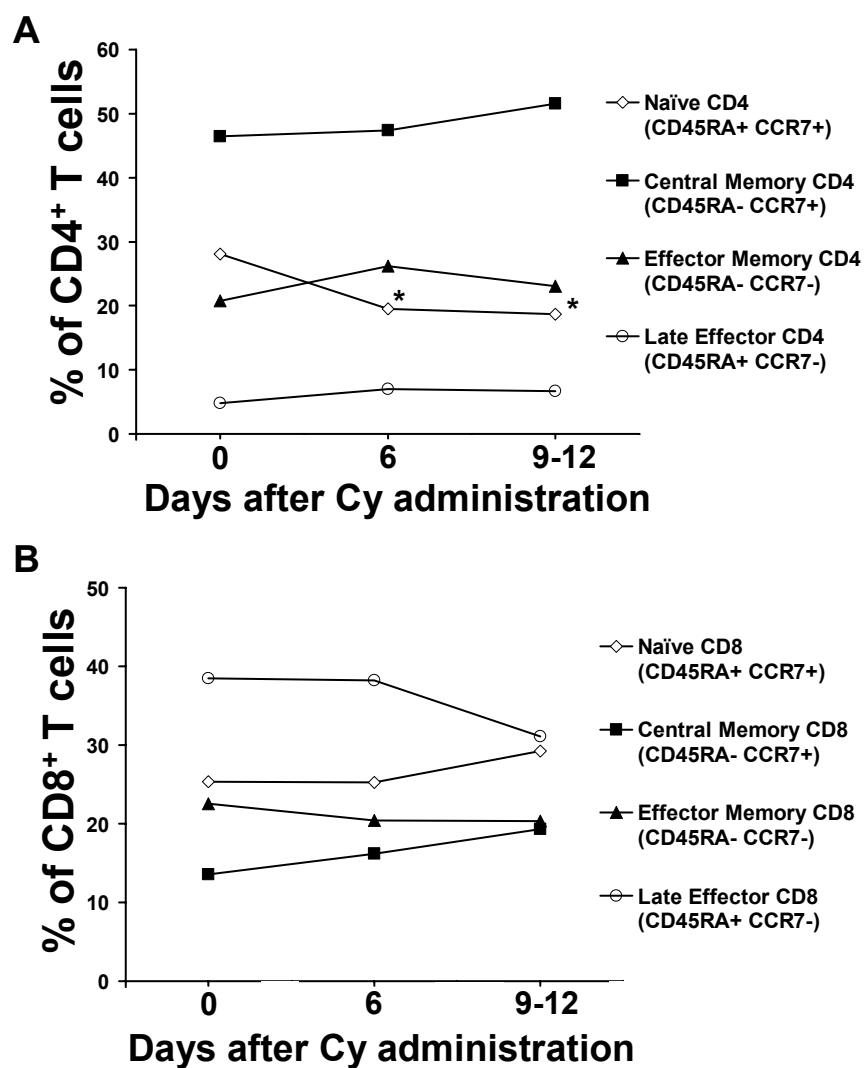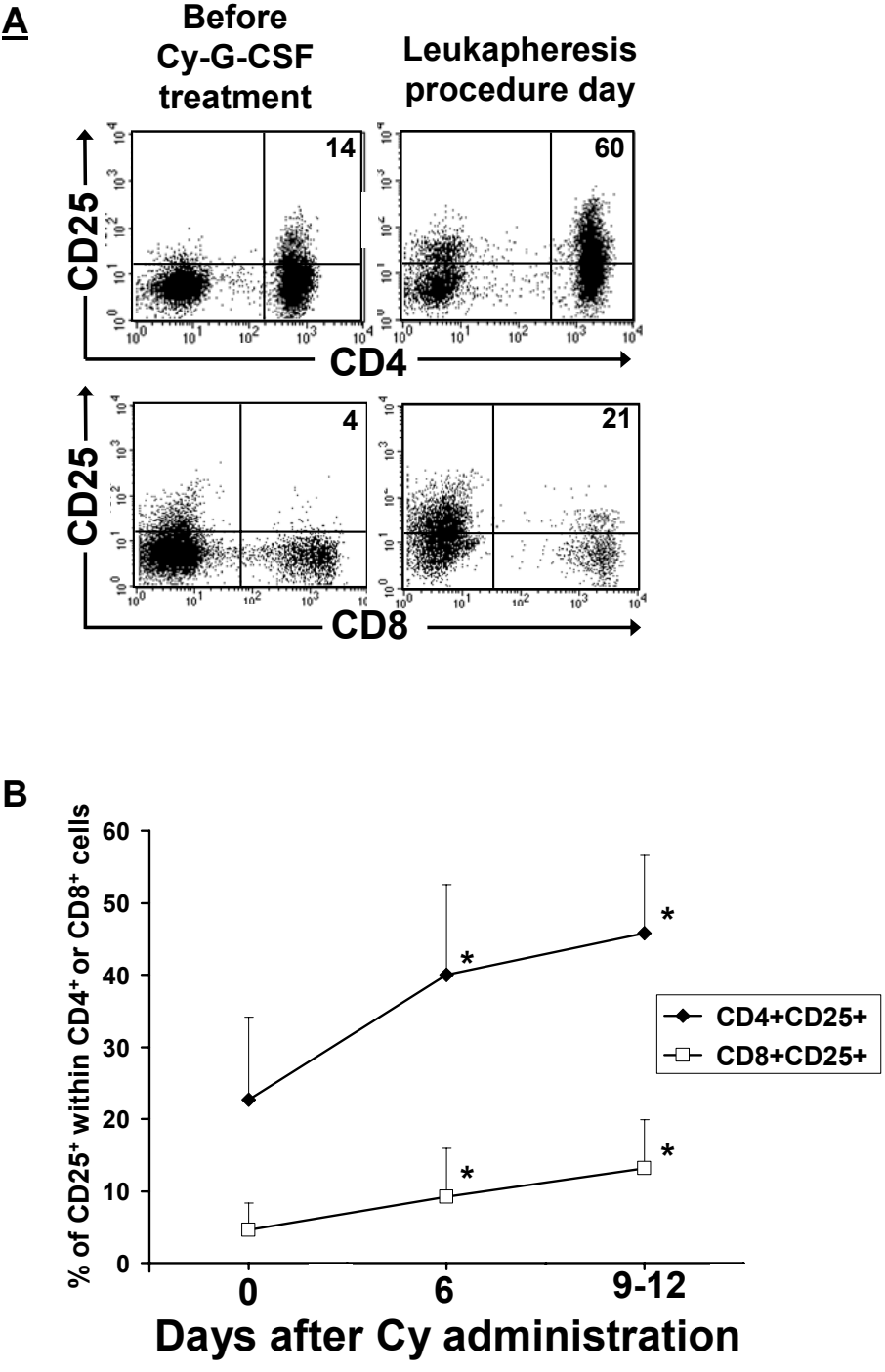
FIGURE 1

**A**



**B**

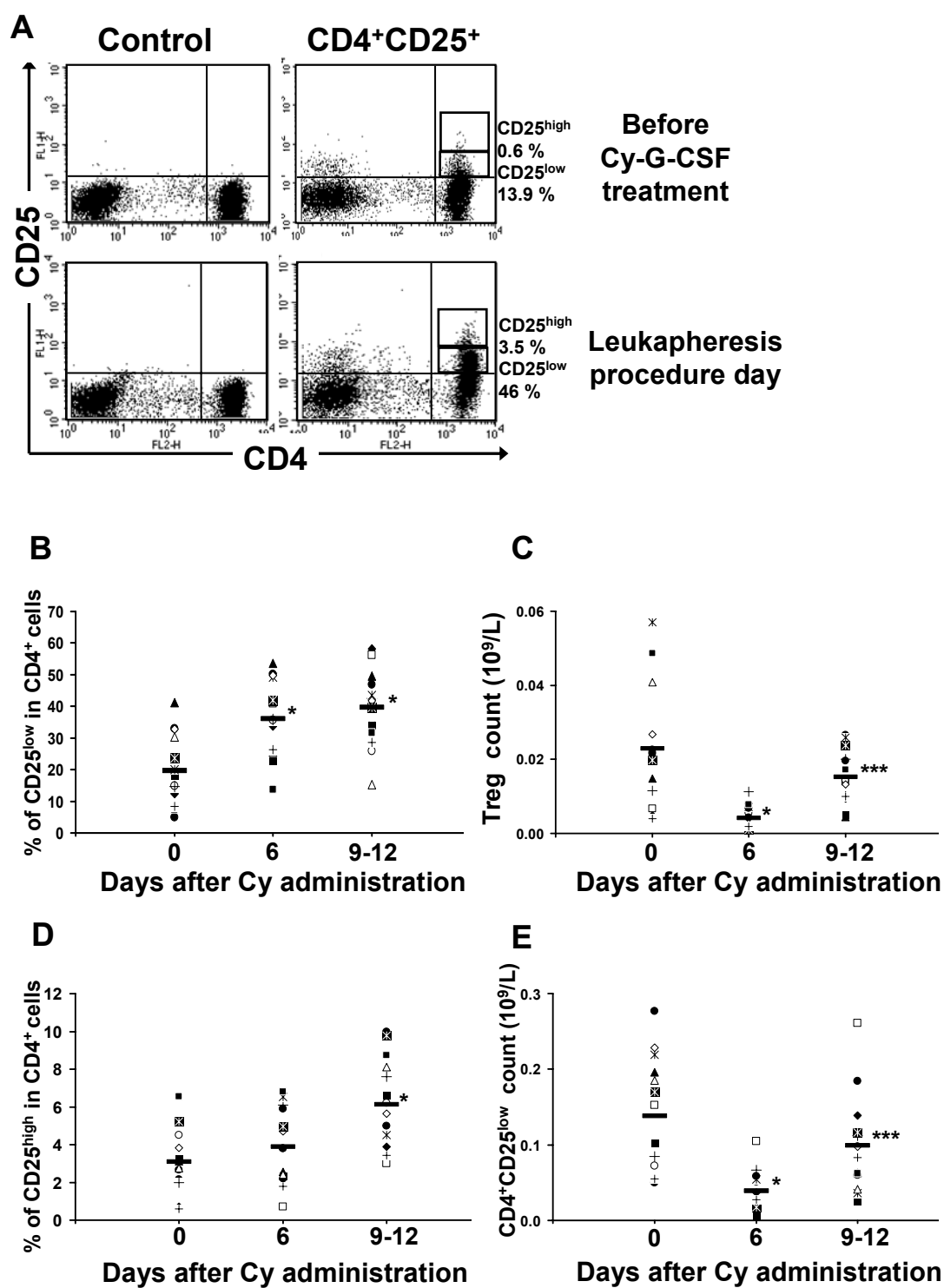

FIGURE 2

**A**

Before
Cy-G-CSF
treatment

Leukapheresis
procedure day



**B**



FIGURE 3

**A**

Control    CD4⁺CD25⁺

CD25 (FL1-H) vs CD4 (FL2-H)

CD25^high 0.6 %
CD25^low 13.9 %

**Before Cy-G-CSF treatment**

CD25^high 3.5 %
CD25^low 46 %

**Leukapheresis procedure day**

**B**

% of CD25^low in CD4⁺ cells

Days after Cy administration

**C**

Treg count (10⁹/L)

Days after Cy administration

**D**

% of CD25^high in CD4⁺ cells

Days after Cy administration

**E**

CD4⁺CD25^low count (10⁹/L)

Days after Cy administration

FIGURE 4

**A**

Control    CD4+CD25+

CD4+CD25high
CD4+CD25low
CD4+CD25-

CD4+CD25-    CD4+CD25low    CD4+CD25high

97%    90%    98%

**B**

*P*=.03    *P*=.03

Relative *FOXP3* expression

CD4+
CD25high    CD4+
CD25low    CD4+
CD25-

Pt1
Pt2
Pt3
Pt4
Pt5
Pt6

**C**

*P*=.03

Relative *CTLA-4* expression

CD4+
CD25high    CD4+
CD25low    CD4+
CD25-

Pt1
Pt2
Pt3
Pt4
Pt5
Pt6

**D**

*P*=.05    *P*=.03

Relative *GITR* expression

CD4+
CD25high    CD4+
CD25low    CD4+
CD25-

Pt1
Pt2
Pt3
Pt4
Pt5
Pt6

**E**

CD4+CD25-    CD4+CD25low    CD4+CD25high

12    27    97    CTLA-4

12    31    64    GITR

FIGURE 5

32

**A**



**Post-Cy-G-CSF CD4+ T cells**

**B**



**Pre-Cy-G-CSF CD4+ T cells**

FIGURE 6